ALANYL-GLUTAMINE HAS NO EFFECT ON EPIDURAL FIBROSIS IN A POST-LAMINECTOMY RAT MODEL

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ABSTRACT

Epidural fibrosis following spinal surgery is common, and subsequent reoperations are more technically challenging with higher complication rates. A safe and effective therapeutic solution to this difficult clinical problem has yet to be realized. Previous research has demonstrated the effectiveness of alanyl-glutamine in reduction of peritoneal adhesions in a rat abdominal sepsis model. I hypothesized that alanyl-glutamine may be similarly efficacious in minimizing epidural fibrosis in a rat laminectomy model.

Rats were randomized into three groups: no surgery, laminectomy/normal saline and laminectomy/alanyl-glutamine ($1g\ kg^{-1}$). The surgical groups underwent a lumbar laminectomy with instillation of either normal saline or alanyl-glutamine into the peridural space. Thirty days after surgery, the rats were euthanized and the spinal columns prepared for histological evaluation. A blinded veterinary pathologist and a less experienced student independently graded the extent and maturity of epidural fibrosis.

The laminectomy model was an effective model for epidural fibrosis formation. Rats that underwent laminectomy demonstrated significant fibrosis compared to control animals ($p<0.001$). However, there was no significant difference in histological grade of fibrosis between normal saline and alanyl-glutamine treatment groups ($p=0.83$).

Based on this study, alanyl-glutamine does not appear to have an effect in reducing epidural fibrosis at a histological level. It is possible that alanyl-glutamine may have an effect that is not detectable using this model, in which case further studies with a more sensitive model may be indicated. Resources may be better used elucidating the mechanism by which glutamine acts to reduce adhesions in the peritoneal model and further studies exploiting those mechanisms can be designed.

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FBSS = failed back surgery syndrome
MRI = magnetic resonance imaging
CSF = cerebrospinal fluid
PDGF = platelet derived growth factor
TGF-β = transforming growth factor-beta
ECM = extracellular matrix
° C = degrees Celsius
rpm = rotations per minute
μm = micron

1. INTRODUCTION

1.1 Epidural fibrosis following surgery: defining the problem

The earliest reference to surgical decompression of the spinal canal was described by Paulus of Aegineta (625-690 AD). Further development of surgical techniques halted until the advent of antisepsis and anesthesia in the 19th century. The first laminectomy in the United Stated is attributed to Alban Gilpin Smith in 1829. However, it wasn't until Mixter and Barr presented their surgical findings regarding ruptured discs at the Annual Meeting for the New England Surgical Society in 1933, with subsequent publication in the New England Journal of Medicine, that lumbar laminectomy and discectomy became one of the most common operations performed by neurosurgeons[1].

Case series presenting the results of lumbar spinal decompression surgery began to be published in the mid-twentieth century[2-5]. In these early reports, up to 19% of patients had persistent or recurrent pain. A small percentage underwent re-exploration and the majority was found to have dense fibrotic adhesions in the epidural space. In these early years of spine surgery before the advent of sophisticated imaging techniques, one could speculate that significant portions of these failures were due to wrong diagnosis and wrong level surgery. However, despite advances in technology and surgical technique, unsatisfactory outcomes following spinal surgery remain a significant problem. Recent literature reviews suggests failure rates of 20-46% for lumbar spine surgery, dependent on the nature of the procedure[6 7]. Applying these numbers to an estimated 28,000 spinal surgeries performed in Canada each year results in an alarming number of unsatisfactory results[8].

The term "failed back surgery syndrome (FBSS)" has been coined to describe this group of patients – those who have unresolved or new onset of symptoms following lumbar spine surgery[9], or whose outcome does not meet the pre-surgical expectations of the patient and surgeon[10]. Rather than a technical failure, the term refers to a failure of the treatment to alleviate the preoperative symptoms. The FBSS population encompasses a heterogeneous patient group with varied preoperative diagnoses who have undergone different, and sometimes multiple, surgical procedures. The myriad of surgical and

nonsurgical explanations for their symptoms present a diagnostic challenge. Chan and Peng proposed a practical classification that considers etiology of FBSS based on preoperative factors (patient factors, procedure selection), intraoperative (technique, incorrect level, inability to achieve aim of surgery), and postoperative (progressive disease, epidural fibrosis, surgical complication, iatrogenic instability)[6]. Of these factors, epidural fibrosis has received particular interest as a potentially preventable factor.

Epidural fibrosis is the expected end result after surgical manipulation of the epidural space, and can be defined as scar formation at the site of surgical access into the spinal canal, in intimate vicinity to and around the origin of the radicular sheath[11]. The full extent of the epidural fibrosis is realized by six months postoperatively[12]. Contrast enhanced magnetic resonance imaging (MRI) remains the best confirmatory test for epidural fibrosis[13], and is often undertaken to investigate alternate explanations for ongoing postoperative pain. The typical appearance is that of a nonspecific enhancing soft tissue mass surrounding the nerve roots and thecal sac at the operative level[14]. The extent and severity of fibrosis is not necessarily dependent on the type of procedure, and can vary between individuals undergoing the same procedure[15-18].

Recognition of epidural fibrosis as a potential problem has led to significant research to characterize the wound healing response and to investigate substances to prevent fibrosis and novel treatment modalities. This effort has been paralleled by refinements in surgical technique and advancements in minimally invasive techniques that minimize tissue disruption. Despite the progress that has been made, the rate of unsatisfactory outcomes following lumbar spine surgery remains unchanged from that reported in the 1950s and there remains a large group of patients who are not helped by surgery. Physicians are ill-equipped to manage this ever-growing population of patients with chronic pain, and epidural fibrosis remains a challenge for surgeons performing revision procedures.

1.1.1 Prevalence

Modern imaging techniques have facilitated detection and diagnosis of a variety of postoperative conditions that may lead to persistent pain following spinal surgery. Imaging remains a useful tool to appreciate the extent of a problem within a population and gauge response to intervention.

In studies of symptomatic postoperative patients, imaging has revealed the prevalence of epidural fibrosis in 46-100%[16 19-21]. Epiduroscopy in a similar population demonstrated significant fibrosis in 91%[22]. These numbers are not surprising, as the formation of epidural fibrosis is a normal response to any surgical intervention that involves manipulation of the epidural space. Some degree of peridural fibrosis will be seen on MRI in the majority of asymptomatic postoperative patients[13 15 16 21-25]. What the data is unable to define for physicians is what percentage of those affected by epidural fibrosis have symptoms that can be attributed to epidural fibrosis.

1.1.2 The relationship between epidural fibrosis and clinical outcome

Epidural fibrosis is frequently associated with unsatisfactory clinical outcomes, with some authors implicating epidural fibrosis as a causal or contributory factor in 20-36% of FBSS patients[21 26 27]. When pain persists or recurs following surgical intervention, contrast enhanced MRI is the diagnostic modality of choice to investigate the etiology of the symptoms. As discussed previously, a significant proportion of post-operative patients will demonstrate epidural fibrosis on imaging. When fibrosis is the sole finding on subsequent investigations or during revision surgical procedures, some surgeons erroneously associate pain with fibrosis. However, establishing causality between fibrosis and clinical outcome demands rigorous investigation that establishes a biologic mechanism that explains the association, clinical studies that consistently support that association and studies that demonstrate alteration of outcome with treatment of fibrosis. The literature to date fails to adequately address each of these requirements, and therefore there remains debate as to whether the presence, location or severity of epidural fibrosis contributes to post-operative patient symptomatology.

The focus of the majority of research conducted to date has focused on prevention of fibrosis and treatment of adhesions, with comparatively little effort expended on exploring the biologic mechanism by which epidural fibrosis may cause pain. The current hypothesis is that the fibrotic material acts as a mechanical tether of the nerve root. Rather than a direct compressive effect[28], it is more likely that fibrosis limits the mobility of an already injured and inflamed nerve root and increases susceptibility to degenerative stenosis and recurrent herniation[29]. Additionally, fibrotic material may impair vascular and cerebrospinal fluid flow, resulting in hypoxia and hypersensitivity of

the nerve root[30 31]. This may explain findings in animals that show increased axonal swelling and electrophysiological conduction blocks in fibrotic areas unrelated to a preexisting lesion or intraoperative iatrogenic damage[28 32]. More research needs to be undertaken to expand upon and validate the current hypotheses.

Clinical studies undertaken to explore the relationship between fibrosis and post-operative symptoms have been unable to demonstrate an association between presence, severity or location of fibrosis and symptomology[15-18 24 33]. Several of these studies have limitations including small sample size[16 24], not defining pain symptoms as radicular[15 24] and premature imaging prior to maturation of fibrosis[33]. However, the studies employing modern imaging techniques and validated outcome measures fail to demonstrate any association between presence or extent of epidural fibrosis and postoperative radicular pain[17 18]. Of the studies claiming an association between postoperative fibrosis and pain, only a single trial examined a suitable sample size and utilized modern imaging techniques[21]. Ross et al state that patients with fibrosis were 3.2 times more likely than patients without fibrosis to develop recurrent radicular pain after discectomy. However, this oft-quoted finding could only be demonstrated on an analysis of a small subgroup, and the majority of those affected by severe fibrosis were asymptomatic. Clinical trials have been unable to explain why some patients with epidural fibrosis remain asymptomatic while others develop severe pain.

Revision surgery in the setting of epidural fibrosis as the sole finding is seldom undertaken as fibrotic areas paradoxically heal with more scar tissue, and outcomes have been poor[34-38]. Interventional techniques such as percutaneous and endoscopic adhesiolysis (a procedure which mechanically lyses epidural adhesions) have been explored as less invasive treatment options for postoperative patients with epidural fibrosis and persistent symptoms. Proponents of the technique claim that high volume lavage mechanically lyses adhesions, reduces local concentrations of inflammatory mediators and facilitates drug delivery to target areas[39]. The sole randomized controlled trial that examined a postoperative population of patients with epidural fibrosis undergoing percutaneous adhesiolysis versus caudal epidural injection suggests clinically significant improvements in both pain and disability scores in the adhesiolysis group[40]. Additionally, a recent systematic review that included trials that looked at more

heterogeneous patient populations found fair evidence that adhesiolysis is effective in treatment of chronic pain following lumbar surgery[41]. However, a biomechanical study suggests that forces transmitted by catheter are incapable of releasing fibrotic tissue[42]. Additionally, a recent study with a placebo intervention arm has demonstrated long lasting clinically significant reductions in pain and disability scores in patients receiving placebo intervention[43]. Furthermore, the majority of the literature on the topic has been published by a small group of researchers, and has compared patient populations with heterogeneous diagnoses utilizing varying treatment protocols. Adhesiolysis is a novel and interesting technique, but has yet to be demonstrated to be an effective treatment option for post-operative epidural fibrosis.

Epidural fibrosis has been implicated as a potential cause of persistent pain following surgery, but the relationship remains unclear. Studies have failed to demonstrate a clear biologic mechanism and clinical studies point to lack of an association between fibrosis and pain. Further research is needed to determine whether there is any benefit of treatment of adhesions. In postoperative patients, it is unlikely that epidural fibrosis is the sole explanation for persistent pain. Pain is often multifactorial and a myriad of other diagnoses and psychological factors likely contribute to poor clinical outcomes.

1.1.3 Revision surgery

The growing aging population has increasing lifestyle expectations, and more people are presenting to the spine surgeon. New surgical techniques have allowed spine surgery to become an option for an increasing number of patients, and there has been a corresponding rise in surgical rates. Unfortunately, although safe and effective, current less invasive techniques have not resulted in decreased complication or reoperation rates[44 45]. Studies suggest stable reoperation rates independent of initial diagnosis or procedure type[46]. Approximately 15% of patients undergoing lumbar surgery will come to reoperation within five years, and 21-34% of these will undergo "repeat revision" surgery[26 47 48]. Of those having one reoperation, approximately one third will require multiple repeat revision surgeries[26 48]. With increasing number of procedures being performed, it is likely that the burden of revision surgery will continue to grow.

There are many reasons why an individual may come to require a revision spine procedure. Failure to achieve or maintain improvement after the initial surgical procedure may result from progressive neurological deficit or deformity, various complications (i.e. pseudarthrosis, infection, cerebrospinal fluid [CSF] leak, implant related), persistent or recurrent debilitating pain or a combination of the aforementioned factors. In the first two of these groups, established surgical decision making pathways guide management of surgically correctable structural or neurological lesions. In the latter, the broad differential diagnosis of persistent or recurrent pain often combined with psychological factors and secondary gain issues, can make diagnosis and management challenging. Therefore, careful evaluation to establish the underlying etiology is essential in every case being considered for revision surgery.

Revision procedures pose additional technical challenges for the spine surgeon. Re-exploration in subsequent spinal procedures is more time consuming, technically challenging and is associated with higher complication rates[49-59]. Dense epidural fibrosis alters the natural tissue planes resulting in an increased risk of inadvertent dural injury and CSF leak[49-52 54-57 59]. Nerve roots that are adherent to fibrotic material are less mobile and are more difficult to safely identify and expose, with increased likelihood of direct or traction-related injury[53 57]. Revision spinal procedures have also been associated with significantly increased operative time, blood loss and infection rates[52 58 60 61]. While the increased complication rate in revision procedures has not been shown to have adverse effects on long term patient outcome, prolonged hospitalization and need for additional procedures imparts additional financial cost to society[52 62 63]. The development of a treatment that could reduce or eliminate epidural fibrosis may simplify subsequent reoperation and prevent complications, thereby resulting in positive economic implications.

Outcomes of revision procedures are highly dependent on the indication for the intervention. If a structural cause for a patient's symptoms can be identified and corrected with revision surgery, outcomes are generally satisfactory. Despite often requiring more invasive procedures and having higher complication rates, revision patients demonstrate equivalent improvements in outcome to patients undergoing a primary procedure[52 64 65]. This is unfortunately not the case in FBSS patients lacking a

structural explanation for their pain. In cases where a structural lesion is not identified, epidural fibrosis is often the sole finding, and reoperation in this setting has disappointing results[9 35 37 66]. For this reason, experts agree that reoperation in the setting of epidural fibrosis as the sole finding is generally contraindicated[26 67].

1.2 Phases of wound healing

Wound healing is the complex and dynamic process that results in the restoration of anatomical continuity and function after an injury[68]. Fibrosis is the normal end result of the wound healing process, and occurs in the setting of both surgical and traumatic wounds. Wound healing in humans exists on a clinical spectrum, and normal healing demands equilibrium between fibroblastic proliferation and remodeling. Occasionally, excess fibroplasia results in pathologic healing in the form of adhesions, keloid scars and contractures. Inadequate healing can also occur, leading to tissue breakdown, dehiscence and chronic skin ulceration. These challenging clinical problems can be difficult to treat and are associated with significant morbidity. Solutions can only be achieved with a thorough understanding of the basic mechanisms that underlie the wound healing process.

The healing process is well studied and involves a complex series of events. The process of acute wound repair differs little from one tissue to another and is generally independent of the form of injury[69]. Four distinct but overlapping stages have been recognized, including hemostasis, inflammation, proliferation and remodeling[70].

1.2.1 Hemostasis

The initiation of wound healing begins the moment the tissue architecture is disrupted. Blood extravasates into the wound, and platelets come into contact with exposed collagen. This results in platelet degranulation and release of clotting factors and cytokines. Clotting factors allow hemostasis and the formation of a fibrin clot in the wound. Cytokines, including platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β), attract inflammatory cells to the wound[71]. The fibrin clot acts as a scaffold for the migration of inflammatory cells into the wound, and their arrival signals the start of the inflammation phase.

1.2.2 Inflammation

The immune response is critical to wound healing, and involves several types of cells and many mediators, which act in concert to achieve a successful outcome. Neutrophils are the first leukocytes to arrive at the wound and are present within six hours, peaking in concentrations at 24-48 hours post-injury[72]. They function to phagocytize dead cells, bacteria and debris and to release cytokines and chemoattractants that encourage infiltration of macrophages. An unknown stimulus results in apoptosis of the neutrophils, and the infiltrating macrophages ingest the neutrophils and take over their phagocytic role[73].

Macrophages are derived from circulating monocytes and reach peak concentration in the wound at 72 hours post injury[69]. In addition to replacing the neutrophil as the main phagocytic cell, they also play a critical role in the release of cytokines and growth factors to orchestrate the repair process[71]. These cytokines recruit other cells, including additional macrophages, lymphocyte, and fibroblasts to the wound. In the absence of significant contamination the inflammatory phase is very short, lasting one to three days after injury[74]. However, the modulatory role of macrophages is maintained, and they can be detected in the wound after inflammation has ended and until the healing process is complete[69].

1.2.3 Proliferation

T-lymphocytes arrive at the wound approximately one week post-injury and signal the transition to the proliferative phase of healing[75]. Although essential to wound healing, the lymphocytes' role has not been fully defined[76]. In addition to preventing infection, they also appear to play a regulatory role. Lymphocytes exert their various influences through the secretion of lymphokines, which have been shown *in vitro* and *in vivo* to exert both stimulatory and inhibitory effects on fibroblast activity and collagen synthesis[77].

The proliferation phase is characterized by the appearance of the fibroblast and the formation of granulation tissue in the wound. Cytokines produced by macrophages trigger differentiation of fibroblasts from local mesenchymal cells. By the third day post-injury, there is a significant expansion of the fibroblast population, and peak numbers are reached by the seventh day[78]. Fibroblasts produce metalloproteinases that facilitate their

movement and rapidly replace the provisional fibrin network with the extracellular matrix (ECM)[71]. The ECM is rich in type III collagen, fibronectin and hyaluronic acid, and is gradually replaced with type I collagen[79 80]. This matrix supports further fibroblast migration, angiogenesis and granulation tissue[80]. Mechanical stress at the wound and the influence of TGF-β induce myofibroblasts to differentiate from fibroblasts, which assists with wound contraction and closure of the granulated wound.

1.2.4 Remodeling

Reorganization of the provisional collagen network begins almost as soon as it is laid down. Collagen that was previously laid down in a random fashion is reorganized by fibroblasts into thick bundles that are cross-linked along the lines of stress in the tissue[81 82]. Fibroblast cells undergo apoptosis and their numbers begin to decrease at approximately day 14. The down regulation the fibroblast population is not completely understood, but is of importance in certain clinical scenarios, such as hypertrophic and keloid scarring, contractures and chronic non-healing wounds. Despite a down regulation in fibroblast population, collagen synthesis will continue for four to five weeks after injury[73]. Wound tensile strength is slow to increase, with the scar having achieved 20% of final strength at three weeks. Although scar remodeling continues for months to years after injury, even in a mature scar only 80% of tensile strength is ultimately restored[83]. Complete restoration of normal tissue architecture is never achieved[84].

1.3 Review of literature on previously investigated potential antifibrotic agents

Coincident with early reports on failed lumbar surgery and the suggestion that epidural fibrosis may play a contributory role, studies began to emerge investigating ways to ameliorate epidural fibrosis formation. After fifty years of research, potential treatments can be grouped into two broad categories: natural or synthetic substances that aim to act as an interpositional barrier between the neural elements and fibrotic tissue, and newer techniques that aim to modulate the immune system and the inflammatory response. Each of these categories has had contenders that demonstrate limited success, but all have failed to make the leap to more widespread clinical use.

The very first studies concentrated on the initial stages in the wound healing process – the extravasation of blood into the surgical bed, and the migration of

fibroblasts. Although little was understood about the wound healing process at the time, it was generally accepted that fibrotic tissue forms as a result of fibroblast migration from posterior tissues (paraspinal muscles, ligamentum flavum, posterior longitudinal ligament)[85 86]. The focus of this early research was on materials that could act as a physical barrier between the migrating fibroblasts and the neural structures.

Researchers recognized that the intimate relationship between any barrier substance and surrounding neural structures would necessitate that substance to be inert, non-immunogenic and non-toxic. Ideally, it would also be easy to place at the time of procedure, and result in little to no patient morbidity. Meeting these criteria, and one of the first interpositional materials to be investigated, was autogenous fat graft. The mechanism by which free fat graft alters the formation of fibrosis has not been established. Theoretically, the fat graft occupies the operative space to act as a physical barrier against hematoma and fibroblasts[87]. Initial success at reducing fibrosis in animal models has not been consistently reproduced in human studies, and it is unlikely that fat grafting has any effect on clinical outcomes[88]. Subsequent reports on graft migration resulting in cauda equina syndrome or nerve root compression and questions about efficacy have led to a decline in clinical use[89-93].

Research efforts continued to focus on interpositional barrier materials for the remainder of the 20th century. Numerous other synthetic interpositional materials were investigated, including hemostatic agents[94 95], silastic membranes[96], sodium hyaluronate[97], synthetic polymers[98-101], carboxymethylcellulose[102] and bioabsorbable antifibrotic gels[103 104]. Of these, only carboxymethylcellulose gel and polytetraflouroethylene (GoreTex™) have demonstrated positive results in animal studies and have been proven non-toxic in human trials[105-107]. High quality human trials demonstrating objective evidence of a decrease in fibrosis with clinically significant differences in outcomes are lacking.

Over the last fifteen years an improved understanding of the wound healing process has resulted in a shift in research focus to agents that modulate the immune system, the inflammatory response and fibroblast proliferation. Corticosteroids[108], mitomycin C[109-112], hydroxycamptothecin[113], colchicine[114], tissue plasminogen activator[115], methylene blue[116], azithromycin[117], human amniotic membrane[118 119], all-trans retinoic acid[120],

verapamil[121], bevacizuman and 5-flourouracil[122] have been investigated. Of these, mitomycin C and hydroxycamptothecin have had promising anti-fibrotic effects, but predictably, further development has been hindered by problems with wound healing[123 124]. The inflammatory cascade plays an intricate role in wound healing, and the ability to target powerful immunomodulators to just the 'undesirable fibrosis' whilst preserving normal wound healing remains a hurdle to be overcome.

Research on epidural fibrosis has progressed significantly from initial studies investigating autologous fat grafts. Despite intensive research effort, inconsistent results in animal models and concerns about local toxicity and wound healing have not translated into clinical success, and no single agent is currently routinely used to decrease epidural fibrosis following spinal surgery.

1.4 Glutamine

1.4.1 Synthesis and metabolism

Glutamine is the most abundant free amino acid in the plasma[125 126]. Although orally ingested glutamine is readily taken up at the jejunum, the majority is metabolized in the intestine itself by the enterocytes and gut associated immune cells[127 128]. The liver catabolizes the remainder with conversion to glycogen or glucose[129]. The majority of the abundant plasma stores are acquired by *de novo* synthesis at the skeletal muscle[130], where glutamine alone accounts for greater than 60% of the free amino acid pool[131].

Glutamine synthesis and release are precisely regulated. In response to heightened catabolism, glucocorticoid mediated upregulation of the glutamine synthetase gene results in increased formation of glutamine from glutamate and ammonia[132]. Branched-chain amino acids originating from the breakdown of muscle protein, and glutamate acquired from the circulation are metabolized in muscle for glutamine synthesis[128 133]. An amino group is donated to α-ketoglutarate to form glutamate and a branched chain α-keto acid. Glutamine synthetase then catalyzes the formation of glutamine from glutamate and ammonia[133]. The glutamine synthetase enzyme is most abundant in muscle, lung, and adipose tissue, and these form the primary sites of glutamine production[129]. Based on mass, skeletal muscle is the most significant site of production.

The ability to be synthesized *de novo* resulted in glutamine being traditionally classified as a 'nonessential' amino acid. However, during catabolic states glutamine stores are rapidly depleted, and consumption exceeds synthesis. During these periods of physiologic stress, there is a corresponding release of glutamine into the bloodstream[133 134]. Despite this significant release, glutamine depletion has been demonstrated in catabolic states such as critical illness[135 136], major trauma[137-139], sepsis[140 141] and postoperatively[142-144]. The recognition that *de novo* synthesis is insufficient to meet increased demands in certain clinical states has led to a change in nomenclature and glutamine is now considered a 'conditionally essential' amino acid in states of critical illness or injury[76].

1.4.2 Metabolism and mechanisms of action

The critical role of glutamine in cell survival and proliferation was first demonstrated *in vitro* in the 1950s. Experiments in mouse fibroblast and HeLa cell culture demonstrated that glutamine had to be present 10- to 100-fold in excess of other amino acids in culture, and could not be adequately replaced by glutamic acid or glucose[145]. It is now known that glutamine is a precursor or product in multiple metabolic pathways in a variety of cells and tissues within the body, including the small intestine, liver, kidney, immune system and brain. In addition to this metabolic role, glutamine also plays a critical role in the endogenous response to stress. Decades after the importance of glutamine was first recognized, researchers are only beginning to fully appreciate the importance of this nutrient at a cellular and whole-organism level.

Plasma glutamine homeostasis reflects a balance between release from muscle and uptake by the splanchnic bed (intestine and liver). Most of the glutamine produced by muscle and released into bloodstream is extracted by the intestine[146 147]. Glutamine is transported to the mitochondria of the crypt cells of the small bowel, where glutaminase converts it to glutamate and ammonia[146]. The glutamine is then metabolized to α-ketoglutarate to be used in the tricarboxylic acid cycle as fuel for the enterocytes and the gut associated lymphoid cells[148]. The ammonia that is generated during this process is excreted as urea, and so glutamine additionally serves as an important shuttle to dispose of ammonia from the body.

The function of the intestine as a major hub in inter-organ glutamine trafficking is not accidental, as the intestine itself is a major glutamine utilizer. The intestinal lumen

and the mesenteric arterial circulation serve as dual sources to meet intestinal glutamine needs. A significant proportion of glutamine is oxidized as metabolic fuel for the enterocytes and gut associated lymphoid cells[148]. Additionally, glutamine serves as a precursor for N-acetylglucosamine and N-acetylgalactosamine, which are involved in intestinal mucin synthesis and intestinal barrier function[148]. Animal models fail to develop normal gut integrity in glutamine-deplete environments[149], while those supplemented with glutamine demonstrate decreased permeability and improved mucosal integrity[150-154], underscoring the critical role of glutamine in maintenance of intestinal structure and function.

The liver receives the majority of its glutamine supply from the intestine via the portal vein[155]. The liver is unique in that it has zonally localized glutamine synthetase and glutaminase enzymes and is therefore capable of net glutamine uptake or output. In addition to precisely regulating glutamine homeostasis, the zonally localized enzymes act simultaneously in an eloquent system capable of removing all excess ammonia from the circulation[155 156]. The portal blood first contacts the hepatocytes capable of urea synthesis, and any ammonia that escapes urea synthesis is subsequently taken up downstream for glutamine synthesis[156]. During times of low glutamine availability (i.e. acidosis following critical illness or injury), glutaminase activity is decreased, and the glutamine synthetase activity is increased resulting in net glutamine output. By utilizing glutamine synthesis as a secondary system for ammonia detoxification, this unique system ensures systemic ammonia homeostasis.

Renal glutamine metabolism has a fundamental role in maintaining acid-base homeostasis within the body. Under physiologic acid base conditions, the kidneys extract and catabolize very little plasma glutamine[129]. The glutamine that is reabsorbed at the epithelial cells of the proximal convoluted tubule is metabolized by the glutaminase/glutamate dehydrogenase system, with the majority of the generated ammonia released to the bloodstream via the renal vein. A small portion (<30%) is excreted in the urine[157 158]. In this case, a hydrogen ion dissociated from carbonic acid combines with ammonia and is excreted. The remaining bicarbonate subsequently enters the circulation where it plays critical role in acid base balance[159]. During periods of metabolic acidosis, rising plasma glutamine levels result in increased renal glutamine

uptake. A shift occurs to favor urinary excretion of ammonium, which assists with maintenance of acid-base balance[158]. The glutamate that was created by the action of glutaminase ultimately participates in gluconeogenesis. Glutamine is the preferred gluconeogenic substrate for the kidney, and results in approximately 20% of endogenous glucose release to the circulation[160].

Cells of the immune system, including lymphocytes, macrophages and neutrophils have high levels of glutaminase and consequently utilize glutamine at a high rate[161-163]. However, glutamine's role extends beyond that of a fuel source, and the more specific involvement of glutamine in immune system function has only become known relatively recently. Glutamine is involved early and facilitates one of the earliest steps in the response to inflammation and infection – migration of immune cells and immune cell adhesion to the vascular endothelium at the site of inflammation[164]. After the arrival of the neutrophil, glutamine appears to enhance phagocytosis and production of reactive oxygen species, both of which are required for bacterial killing[165 166]. As the macrophages arrive, glutamine has a similar role, enhancing phagocytic activity[167-169]. Additionally, glutamine modulates macrophage cytokine release necessary for lymphocyte migration, as well as facilitation of antigen presentation to T-lymphocytes[170-172]. Activated T cells begin proliferating, utilizing the familiar glutaminase/glutamate dehydrogenase pathway to shuttle α-ketoglutarate into the tricarboxylic acid cycle to generate fuel. Additionally, they convert glutamine to purine and pyrimidines for DNA and RNA synthesis[173]. The proliferation and differentiation of β-cells is less well studied, but also appears to be glutamine dependent[174].

In addition to its role in metabolic function of multiple organs and the immune response, glutamine also plays an important role in the endogenous stress response. Under physiologic conditions, metabolically active cells are constantly generating and are exposed reactive oxygen and nitrogen species[175]. This is magnified following critical illness or injury, and overwhelms the cellular antioxidant capacity[176]. These reactive species may interact with proteins, lipids and nucleic acids to disrupt their integrity and function[177]. The most important intracellular antioxidant response is the glutathione system, which eliminates these radicals via reduction[178]. Glutamine's role in this system is as a precursor for glutamate, which is required for glutathione synthesis. An additional

family of proteins, the heat shock proteins, specifically assist with protein renaturation and degradation[176]. They also appear to decrease plasma concentration of proinflammatory cytokines[179]. Enhanced heat shock protein expression has been shown to be cytoprotective in a wide variety of experimental injury models, and glutamine has been demonstrated to be a potent enhancer in multiple organs and cell types[179]. The mechanism by which glutamine enhances heat shock protein remains unknown[179].

In addition to the pathways discussed above, significant glutamine metabolism occurs in the brain. Glutamine is the precursor of the neurotransmitters glutamate and γ-aminobutyric acid, provides substrates for energy production in the tricarboxylic acid pathway and also has a critical role in cerebral ammonia homeostasis[180]. While important, these pathways have negligible effect on the response to injury or the post-surgical healing response, and will not be discussed in detail.

1.4.3 Current clinical applications

A significant body of basic science research provides many possible mechanisms by which glutamine supplementation may exert beneficial effects on multiple organ systems. Growing appreciation for glutamine's pivotal metabolic role led to experimental application in a variety of patient populations with challenging nutritional needs. The most encouraging results have been in the critical illness and oncology populations, leading to clinical adoption of specialized nutritional programs.

A large number of investigations have focused on the clinical efficacy of enteral or parenteral glutamine supplementation following critical illness, major surgery or injury. Common to each of these conditions is an increase in glutamine consumption to support immune function, protein synthesis, gluconeogenesis, acid-base balance and antioxidant status. In order to meet these demands, catabolism of the skeletal muscle protein occurs, with transport of the amino acids to the visceral organs and wound site[181]. Continued glutamine consumption results in sustained glutamine depletion in many patients following critical illness or injury[136 182]. This deficiency has been associated with increased mortality in the critical care population[135 183]. Investigators hypothesize that exogenous glutamine supplementation would limit muscle wasting, optimize metabolic and immune functions and improve patient outcomes.

The potential benefit of glutamine supplementation to the critical care population has resulted in multiple randomized controlled trials on the subject. Meta-analyses of these trials suggest modest effects on reduction of infection and hospital length of stay, but remain divided with respect to the existence of a mortality benefit[184-188]. It is concerning, however, that a recent large multicenter trial reported the converse, suggesting increased mortality rates in absence of any observed clinical benefit in glutamine supplemented patients critically ill patients with multi-organ failure[189 190]. Analysis of the conflicting data highlights the heterogeneity in study populations, dosing regimes and method of administration. Current Canadian critical care guidelines reflect this, and the previous strong recommendation for glutamine supplementation has recently been downgraded to one which 'should be considered' when parenteral nutrition is prescribed to the critically ill[191].

The reported benefits of glutamine supplementation in the critically ill prompted interest in application in oncology populations, where adequate nutritional support is linked to quality of life and outcomes[192-194]. As the body attempts to repair damage induced by cytotoxic treatments on rapidly dividing cells, including those of the immune system and gastrointestinal tract, increased glutamine demand rapidly outstrips supply. Similar to the critical care population, the body attempts to augment circulating glutamine through skeletal muscle breakdown, contributing to cachexia. Despite this, glutamine depletion develops over time, with corresponding negative effects on glutamine dependent tissues, resulting in loss of gut barrier integrity, immune system dysfunction, loss of adipose tissue, increased hepatic production of acute phase proteins and insulin resistance[195].

Despite the potential beneficial of glutamine, there was initial reluctance to experiment with supplementation in this patient population. It is known that tumor cells preferentially consume glutamine to supply metabolic pathways that support growth and proliferation, inducing a net flux from host to the growing malignancy[195]. Additionally, higher glutathione levels in tumor cells mediate resistance to chemotherapy and radiation[196 197]. The overriding concern was that glutamine supplementation might stimulate tumor growth and enhance the action of glutathione to convey resistance.

Fortunately, these concerns have not been realized *in vivo*. Multiple studies have failed to show stimulation of tumor growth, and evidence in fact supports a glutamine-mediated decrease in tumor growth[198-201]. In patients undergoing conventional chemotherapy and radiotherapy, glutamine supplementation has been shown to mitigate side effects associated with cytotoxicity[202-208], improve immune cell counts[209 210] and sustain gastrointestinal barrier function[209 211-214]. Potential benefit has also been observed in bone marrow transplant populations with meta-analysis suggesting that glutamine supplemented patients have lower incidence of mucositis, graft versus host disease and infectious complications[215]. Again, relative small numbers of studies with considerable heterogeneity in study populations, dosing regimes and methods of administration limits clinical applicability.

A growing body of clinical evidence suggests a beneficial role for glutamine supplementation in the critically ill and oncology populations. However, further clinical trials are needed to determine which sub-populations benefit the most, and the optimal dosing regime and duration. Until then, strong clinical guidelines will remain lacking, leaving the clinician to weigh the evidence for each unique clinical situation.

1.4.4 Potential role in wound healing

Surgical intervention results in an inflammatory response with increased catabolism and transport of energy, free fatty acids and amino acids to the wounded site and a corresponding fall in plasma glutamine levels[181 216 217]. Adequate post-operative nourishment is essential to support this response, resulting in a cosmetically acceptable wound with maximal mechanical strength with minimal disruption of tissue architecture and maintenance of tissue planes. Given glutamine's critical involvement with the metabolism of cells involved with wound repair, it is reasonable to hypothesize that supplementation to normalize plasma glutamine levels would optimize metabolic conditions for healing.

Animal studies provide a significant amount of indirect evidence of a positive effect of glutamine supplementation on post-operative wound healing. Several studies of enteral glutamine supplementation in a colonic anastomosis model demonstrate enhanced angiogenesis, inflammatory and fibroblast cell counts and collagen deposition with resultant increase in mechanical strength of the anastomosis site[218 219]. The intestinal

healing environment, however, is unlike that of a surgical wound, and effects of enteral glutamine may be due to trophic effects on locally responsive intestinal cells. However, the effects are not merely trophic as positive effects on anastomotic healing can still be demonstrated after parenteral administration[220].

Studies investigating glutamine supplementation in cutaneous models of healing have revealed mixed results. Incisional healing models demonstrate increased collagen deposition and tensile strength[221 222], and an excisional model revealed increased angiogenesis, macrophage and fibroblast infiltration and collagen deposition with resultant decreased time to wound healing[222]. Two other studies utilizing the excisional model failed to show an effect of glutamine supplementation, however the treatment dose was significantly lower than in other studies[223 224].

The majority of clinical trials investigating the effect of glutamine supplementation in surgical populations have focused on broader clinically important outcomes (ventilator time, hospital stay, rates of infection, mortality). A recent meta-analysis revealed positive effects of parenteral glutamine supplementation on reduction of infection related complications and length of stay[187]. Although a reduction in infection related complications might be related to superior wound healing, there are no studies specifically examining the effect of glutamine supplementation on wound healing in a surgical population. Similarly, there are no studies examining specifically a spine surgery population, however experts agree that glutamine supplementation may benefit all postoperative groups at high risk for infection[217].

Although the metabolic effects of glutamine are well described, the exact mechanism by which supplementation affects wound healing remains unknown. Authors who have observed positive effects hypothesize enhanced substrate availability for rapidly dividing cells, improved immune cell function and a heightened inflammatory response[76 218 225]. However, while a robust inflammatory response may be evolutionarily desirable for healing of contaminated wounds, there is some evidence suggesting that a blunted inflammatory response may result in more desirable healing in controlled situations (i.e. non-contaminated surgical wounds). Studies suggest enhanced, and even scarless healing, in mouse lines devoid of inflammatory cells[72]. Additionally, mice with a deletion of the gene responsible for the SMAD family of proteins that mediate TGF-β

signaling and inflammatory cell activation demonstrate reduced influx of inflammatory cells and accelerated cutaneous wound healing[226]. This is consistent with research in fetal healing, which has minimal or no scar formation prior to 24 weeks gestation[227]. Fetal wounds demonstrate an attenuated inflammatory response[228-230], and there is a positive correlation between increased inflammatory cell activity in older fetuses and increased scarring[231]. If a blunted inflammatory response results in superior wound healing, then perhaps the effect of glutamine supplementation is antagonistic to healing with minimal scar, instead supporting exuberant collagenization at the wound site.

Post-operative glutamine supplementation has beneficial effects on clinically important outcomes, but strong evidence supporting a direct benefit to wound healing is lacking. Further research need to be undertaken to characterize the optimal healing environment required to achieve maximal wound mechanical strength with minimal tissue disruption, recognizing that the ideal environment might be unique to each clinical situation. Only with the knowledge of the mechanisms underlying the optimal healing response can research be undertaken to attempt to modulate that response with targeted supplementation of key nutrients or factors.

2. HYPOTHESIS AND RESEARCH OBJECTIVES

Given the critical role of glutamine in the body's response to trauma and wound healing pathways, and its effectiveness in reduction of peritoneal adhesions in a rat abdominal sepsis model[232], it is reasonable to hypothesize that glutamine may be similarly efficacious in reducing fibrosis in other clinically relevant areas. This study tests the hypothesis that peridural application of glutamine will result in decreased formation of epidural fibrosis in rats following laminectomy. The precise mechanism of glutamine's effect is unknown, but is presumed to be through local modulation of the cells involved in wound healing.

To test this hypothesis, the research objectives were:

1. To determine whether there is any significant difference between histological extent and maturity of epidural fibrosis in rats having undergone laminectomy and instillation of normal saline versus those who were treated with alanyl-glutamine.
2. To determine whether there are any adverse effects of peridural alanyl-glutamine application on the animal or the healing wound.

3. MATERIALS AND METHODS

3.1 The animal model

Peridural fibrosis has been consistently produced in rats, rabbits and dogs[85 87 233 234]. The course of fibrosis development following laminectomy in the rat is similar to that observed in other animal models[233]. However, when compared to other models, the use of the rat model is advantageous with respect to its relative low cost, ease of availability and care.

In this study, the animals were followed for a period of 30 days following treatment prior to euthanasia and tissue preparation. This allows adequate time to observe the animal for any adverse effects of the treatment substance, monitor wound healing and allow for fibrosis formation. In the rat model, fibrosis at the laminectomy site is evident as early as 15 days. Beyond 30 days, new bone may form over the laminectomy defect, making it more difficult to identify during tissue harvest[233].

3.2 Animal preparation

All care and treatment protocols were approved by the University of Saskatchewan Animal Care Committee prior to experimentation. Animals were treated in accordance with the Canadian Council on Animal Care Guidelines.

Adult male Wistar rats (Charles River, Laval, PQ, Canada) were acclimatized for one week prior to surgery. Rats received standard rat chow and water *ad libitum* and were kept in a 12:12 hour light-dark cycle at 25° Celsius.

Anesthesia was induced with inhalational isoflurane 2% (MTC Pharmaceuticals, Cambridge, ON, Canada) in oxygen at 5 liters min^{-1}. Animals were maintained under anesthesia using inhalational isoflurane 2% at 2-3 liters min^{-1}. Preoperative analgesia was provided with subcutaneous buprenorphine 0.05 mg kg^{-1} (Western Drug Distribution Center, Edmonton, AB, Canada). Each rat was marked with an identifying ear tag. The

rat was placed in the prone position, and the dorsal skin overlying the operative site was shaved and sterilized with Hibitane® (chlorhexidine gluconate, Ayerst Laboratories, Montreal, ON, Canada) and 70% ethanol.

3.3 Groups and treatments

Rats were randomized into two groups (Table 1): a surgical control group who underwent laminectomy with instillation of normal saline (n=34), and a treatment group that underwent laminectomy with alanyl-glutamine instillation 1g kg^{-1} (n=36). Dose was selected based on previous research[232 235 236], and approached the maximal soluble concentration[237]. A weight-based calculation was performed to determine the volume of alanyl-glutamine 400 mg ml^{-1} (AdeTherapeutics Inc., Saskatoon, SK, Canada) to be instilled to achieve a dose of alanyl-glutamine 1 g kg^{-1}. This was performed for all rats, therefore rats receiving normal saline received equivalent volumes. In addition, 6 animals underwent no procedure and were used as histological controls.

Table 1.1: Treatment groups.

	Control - no surgery	Laminectomy + normal saline	Laminectomy + alanyl-glutamine ($1\ g\ kg^{-1}$)
Total	6	34	36

3.4 Surgical procedure

Standard sterile surgical techniques were used. Using anatomic landmarks, a dorsal midline incision was made from the fifth lumbar vertebra (L5) to the second sacral vertebra (S2). The paraspinal muscles were bluntly dissected away from the spinous processes and retracted. Using a dissecting microscope and rongeur, a bilateral total laminectomy of a lumbosacral vertebra was completed laterally to the level of the pedicle. The ligamentum flavum (the elastic ligament that connects adjacent vertebrae) and epidural fat were removed to expose the dura mater (the outermost membrane enveloping the spinal cord). Throughout the procedure, hemostasis was maintained by irrigation with saline and compression with surgical pads. Bipolar cautery, bone wax or other hemostatic agents were not used.

The treatment substance was instilled into the laminectomy site just prior to wound closure. In all groups, fascia at the laminectomy site was marked with a non-absorbable suture to facilitate harvest of pathological specimens. Wounds were closed by approximating the paraspinal muscles, fascia and subcutaneous tissues using 3-0 Vicryl® absorbable sutures. The skin was closed using staples. The rat received 2 mL of lactated Ringers solution subcutaneously to restore lost bodily fluids. Post-operative analgesia involved a tapering dose of subcutaneous buprenorphine every 12 hours for three days. No prophylactic antibiotics were used. The rats were observed for postoperative complications, and were monitored daily for signs of sensory deficit distal to the laminectomy site. Sutures were removed seven days following surgery.

3.5 General necropsy and tissue harvest

Animals were killed 30 days after surgery under general anesthesia (1.5-2% isoflurane in oxygen with a flow rate of 1.5 liters min^{-1}). Within two hours of euthanasia, the rats underwent necropsy and the major organ systems were examined macroscopically. The lumbosacral portion of the spinal column was removed *en bloc* and fixed in 10% neutral buffered formalin for 7 days while on a shaker at 60 rotations per minute (rpm). This was followed by slow decalcification process in 20% formic acid for 84 hours, also on a shaker at 60 rpm.

3.6 Tissue preparation

The surgical laminectomy site was identified, and three blocks were trimmed: one section at the marked site of laminectomy, and two sections 2.5 mm cranial and caudal to the marked site (Figure 1). The trimmed blocks were sectioned (5 micron[μm] thick) and stained with Masson trichrome technique.

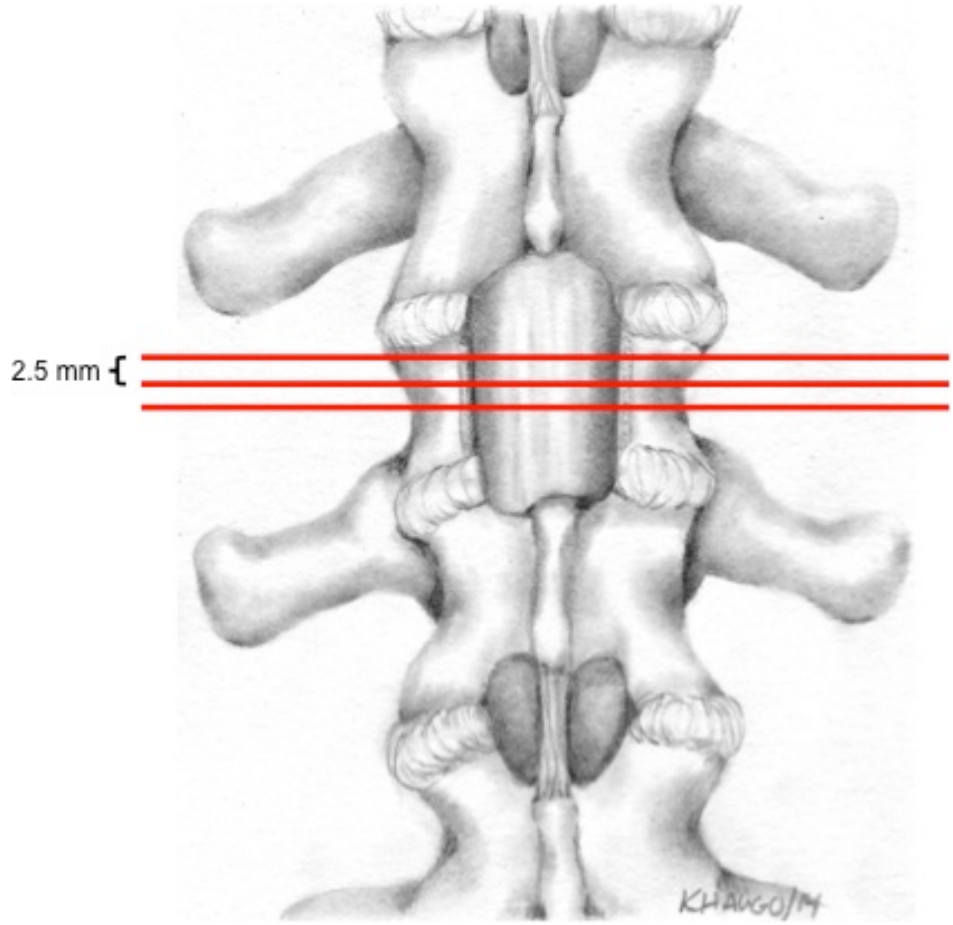

Figure 3.1: Sectioning technique.
Sections were taken 2.5 mm cephalad and caudad to the laminectomy site, marked by a nonabsorbable suture.

3.7 Histology

3.7.1 Masson Trichrome staining

All sections were stained with Masson trichrome technique[238]. This technique involves sequential exposure of the tissue sample to stains with varied molecular size, resulting in differential take up of the stains by tissue components that have variable permeability[239]. This results in nuclei staining blue-black, muscle and cytoplasm pink-red, and connective tissue blue. Masson trichrome technique is particularly useful for differentiation of collagen, and therefore fibrosis, with immature collagen staining a paler blue in contrast to darkly stained mature fibers.

3.7.2 Hematoxylin & Eosin staining of selected specimens

After Masson Trichrome staining, several samples that appeared to be affected by processes that may independently result in fibrosis (i.e. foreign body, pyogranulomatous inflammation, hematoma) were identified. To further characterize these processes, subsequent sections were stained with hematoxylin and eosin and examined with regular and polarized light.

Sections were stained using Harris hematoxylin and eosin[238], which results in hematoxylin staining nuclear elements and chromatin blue, and eosin staining the connective tissue and cytoplasm pink-red.

3.8 Analysis of histological preparations

Each section was examined under a light microscope (Olympus 2X and 4X/0.10 objective, 20X and 40X total magnification). Two digital microphotographs (12.5X and 20X magnification) were taken for each slide. Each section was independently evaluated at two time points by two raters: an anatomic veterinary pathologist and the author. The treatment from which the histological sections originated was unknown to both of the evaluators so as to avoid bias in analysis. Images presented in this text are representative sections reflecting the whole dataset.

3.8.1 Grading of specimens

The sections were graded on a subjective scale evaluating extent and maturity of epidural fibrosis (Figure 2). Grade 0 represented no fibrosis; Grade 1 represented minimal, immature fibrosis; Grade 2 represented moderate fibrosis with mixed immature and mature areas; Grade 3 represented dense, mature fibrosis covering the majority of the laminectomy defect; Grade 4 represented dense, mature fibrosis occasionally disrupting tissue architecture. The grading scheme was a modified scheme based on that formulated by He et al[233].

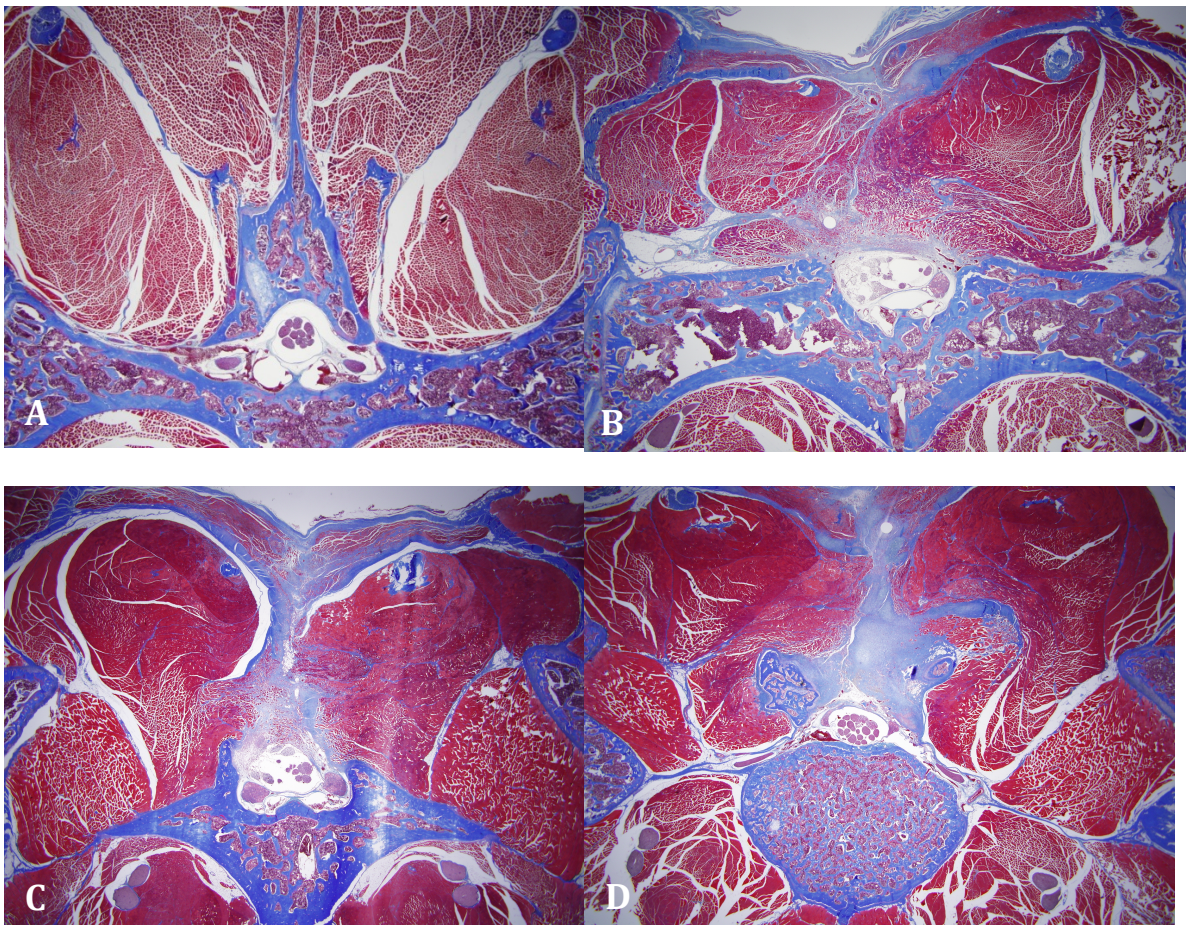

Figure 3.2: Histological grading scheme.
Semi-quantitative evaluation of epidural fibrosis following lumbosacral laminectomy. **(A)** Grade = 0 (control rat); **(B)** Grade = 2; **(C)** Grade = 3; **(D)** Grade = 4. Masson's trichrome stain, 20X magnification.

3.9 Statistical analysis

IBM SPSS Statistics software version 22.0 (IBM Corp., Armonk, N.Y., USA) was used for statistical analysis. Of the three sections obtained for each animal, the grade at the section demonstrating a complete laminectomy was used for statistical analysis. If there was a discrepancy in the grade assigned at the site of complete laminectomy for the first and second pathological readings for an animal, the higher grade was used. Fisher's exact test was used to compare the grade of epidural fibrosis between the treatment groups. The level of statistical significance was defined as $p<0.05$.

The intra-rater reliability was assessed by calculating Cohen's unweighted kappa

value using 104 pairs of independent observations performed by two raters in 52 rats. The inter-rater reliability was assessed by calculating Cohen's unweighted kappa value using 104 pairs of observations performed by two raters in 52 animals, comparing scoring for first grading of each section.

4. RESULTS

4.1 Surgical time

The mean operative time across all treatment groups was 25.4 ± 9.4 minutes. The mean operative time in the normal saline treatment group was 26.2 ± 10.6 minutes, and in the alanyl-glutamine treatment group 25.6 ± 7.9 minutes (Figure 4.1). There was a statistically significant difference in the mean operative time of the first 50% of procedures performed (31.3 ± 2.8 minutes versus 18.9 ± 2.2 minutes, $p<0.001$).

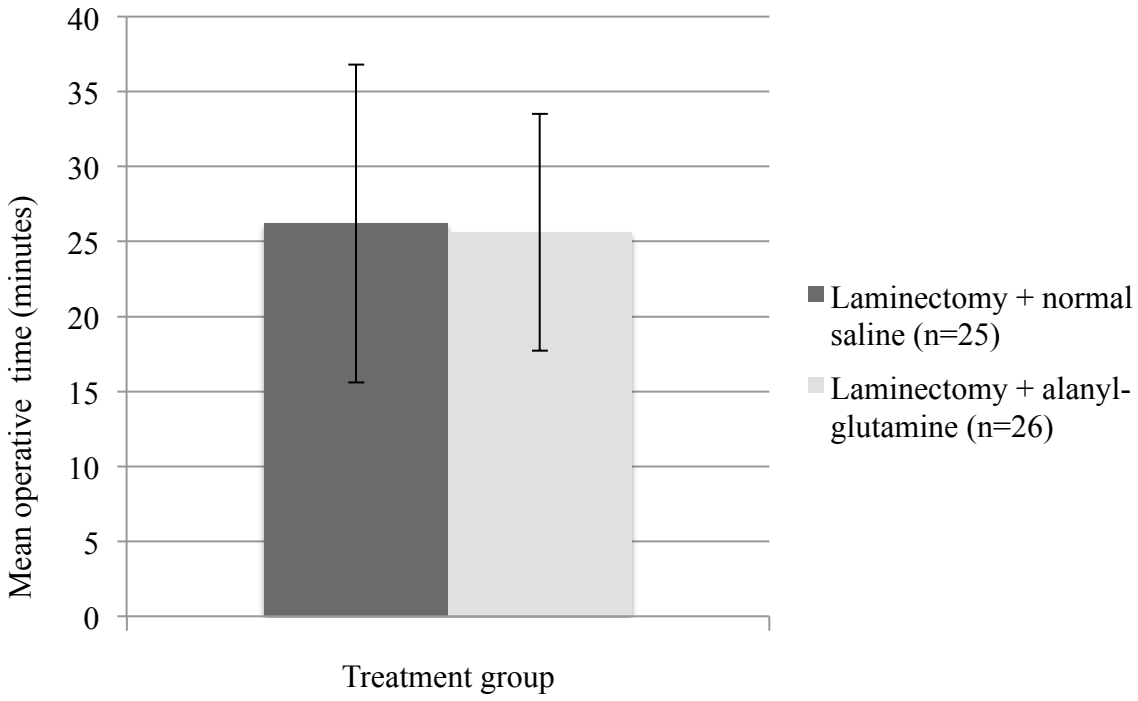

Figure 4.1: Mean operative time within treatment groups.
Data expressed as mean, error bars represent ± standard deviation.

4.2 Intraoperative complications

There were no intraoperative deaths.

There were five intraoperative dural breeches (two in the surgical control group and three in the alanyl-glutamine group), and three intraoperative nerve root injuries (one in the surgical control group and two in the alanyl-glutamine group).

Intraoperative complications are tabulated in Figure 4.2.

4.3 Postoperative complications

There were two postoperative deaths. One (in the surgical control group) occurred on the third postoperative day, and was likely related to postoperative hemorrhage. The second (in the alanyl-glutamine group) occurred on the sixth postoperative day and was unexplained.

One animal in the treatment group had an unexplained seizure within 6 hours of the procedure. The animal subsequently fully recovered without further intervention. This animal was later excluded from histological analysis for reasons unrelated to the seizure (unable to identify laminectomy site on histological preparation).

There were 15 cases of neurologic deficit, manifested by tail numbness. Eight of these cases were in the surgical control group, and 7 were in the alanyl-glutamine group. Seven cases were transient. Eight cases (five in the surgical control group and three in the treatment group) had not resolved prior to euthanasia.

There were fourteen wound hematoma/hygromas (8 in surgical control group and 6 in alanyl-glutamine group). Four (2 in surgical control and 2 in alanyl-glutamine group) clinically resolved prior to euthanasia. At the time of necropsy, those that had not resolved were found to be organized hematomas or sterile hygromas.

There were no cases of wound infection.

Postoperative complications are tabulated in Figure 4.2.

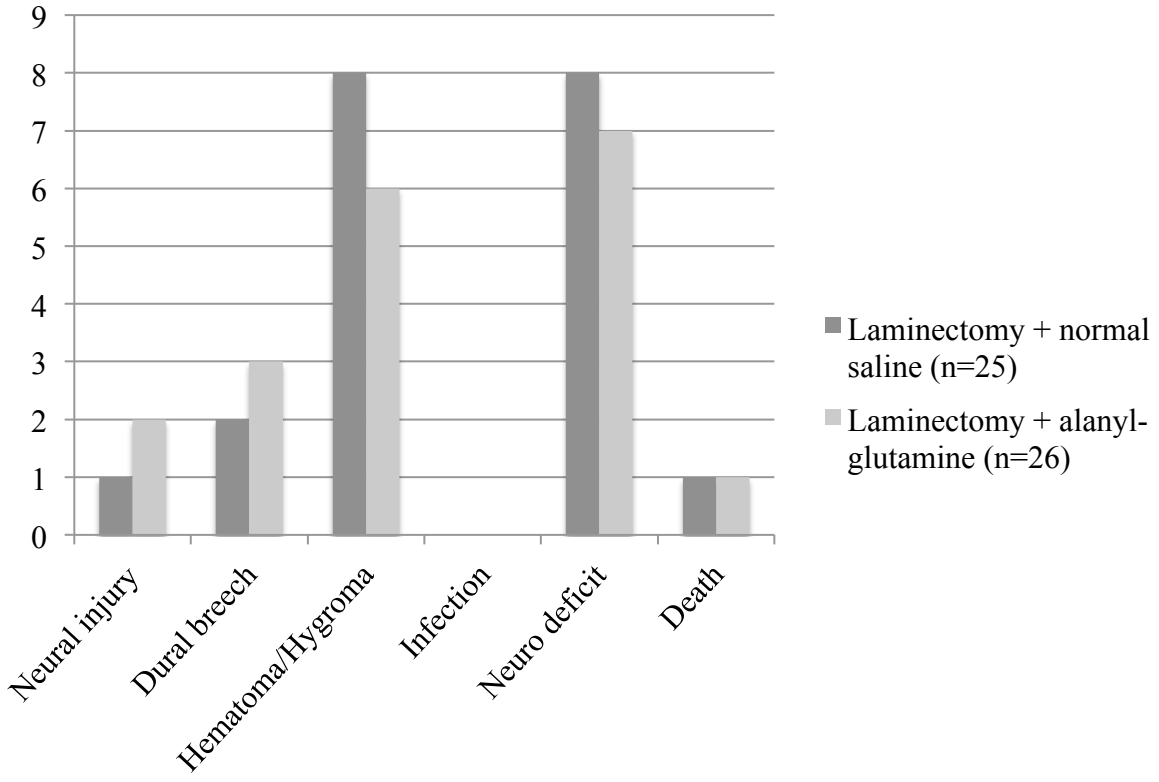

Figure 4.2: Intraoperative and postoperative complications.

4.4 Histological evaluation

4.4.1 Qualitative observations

Based on gross evaluation at necropsy, all rats were healthy, with the exception of one in the alanyl-glutamine group that had uroliths in the urinary bladder and bilateral hydronephrosis. This was likely an incidental finding and would be unlikely to affect the experimental result, however this rat was subsequently excluded from further analysis due to other reasons (see discussion below).

4.4.2 Animals excluded from histological analysis

Upon histological examination, several rats were affected by processes (i.e. pyogranulomatous inflammation associated with foreign material, or adjacent hematoma/hygroma), which may independently result in fibrosis indistinguishable from that induced by surgery (Figure 4.3). These processes were further evaluated with hematoxylin and eosin staining. These processes would interfere with the evaluation of

the effects of therapeutic anti-fibrotic agents, and therefore these animals (nine in the control group and 10 in the alanyl-glutamine group) were excluded from further analysis (Figure 4.4).

Additionally, one rat in the alanyl-glutamine treatment group was excluded from further analysis because despite multiple sections, the area of laminectomy site was unable to be identified (lamina was intact on all sections). This was likely due to migration of the marking suture during antemortem postsurgical fibrosis or post mortem fixation induced muscle contraction.

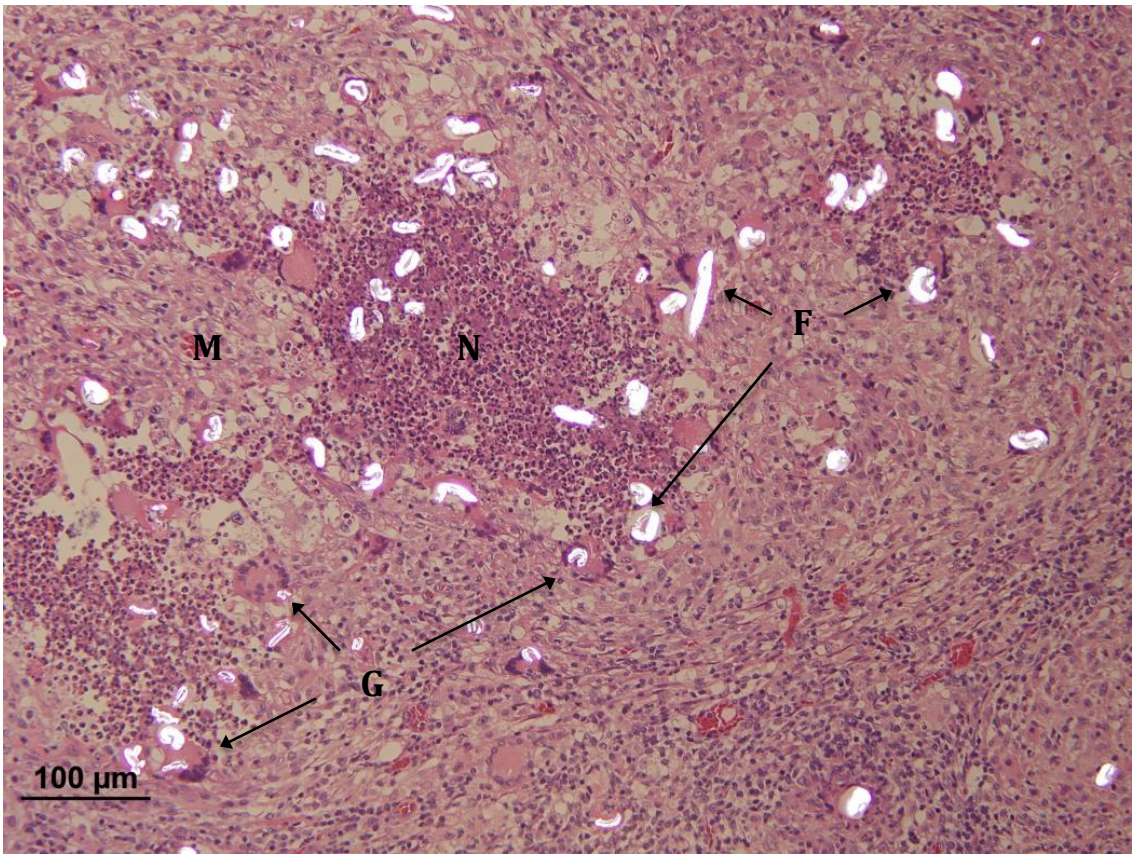

Figure 4.3: Tissue section demonstrating pyogranulomatous inflammation induced by foreign material.
Hematoxylin and eosin stained section exposed to polarized light demonstrating pyogranulomatous inflammation induced by foreign material. Foci of degenerative neutrophils (N), giant cells (G) and macrophages (M), foreign material (F).

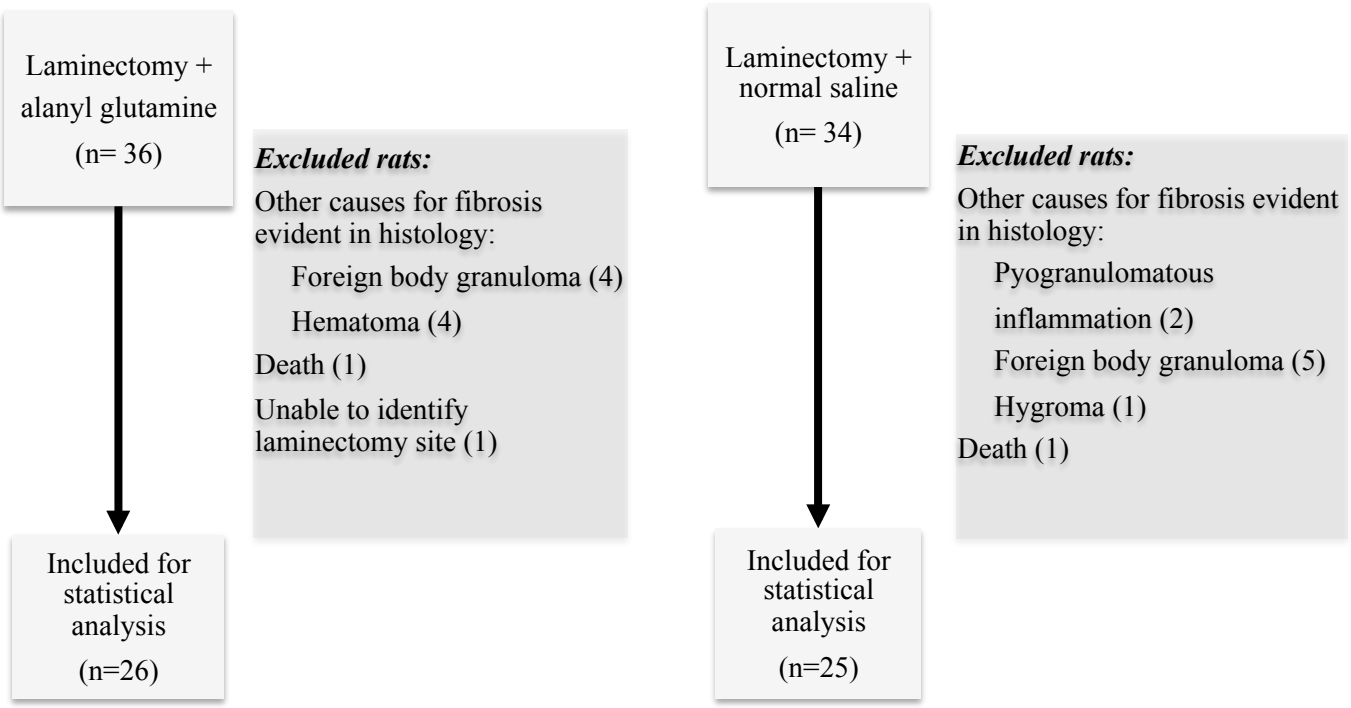

Figure 4.4: Rats excluded from statistical analysis of grade of fibrosis.

4.4.3 Comparison of nonsurgical control and animals having undergone laminectomy

Nonsurgical control rats demonstrated no fibrosis (Figure 4.5). These served as a histological control and a representation of normal tissue architecture and anatomy.

Rats that underwent laminectomy with instillation of normal saline demonstrated exuberant fibrosis in the epidural, deep and superficial epaxial layers. Fibroblastic tissue formed over the laminectomy defect, and extended to adhere to the dura and nerve roots (Figure 4.6). In some cases, fibrosis was so severe that the vertebral canal and nerve roots were indistinguishable from the surrounding fibrotic tissue (Figure 4.7). Fibrosis also occasionally extended to areas adjacent to those that had undergone laminectomy.

Of the 25 rats in the normal saline group, 20 rats had grade 4, four had grade 3 and one had grade 2 epidural fibrosis. Rats treated with normal saline demonstrated significantly more fibrosis than control (nonsurgical) rats ($p < 0.05$).

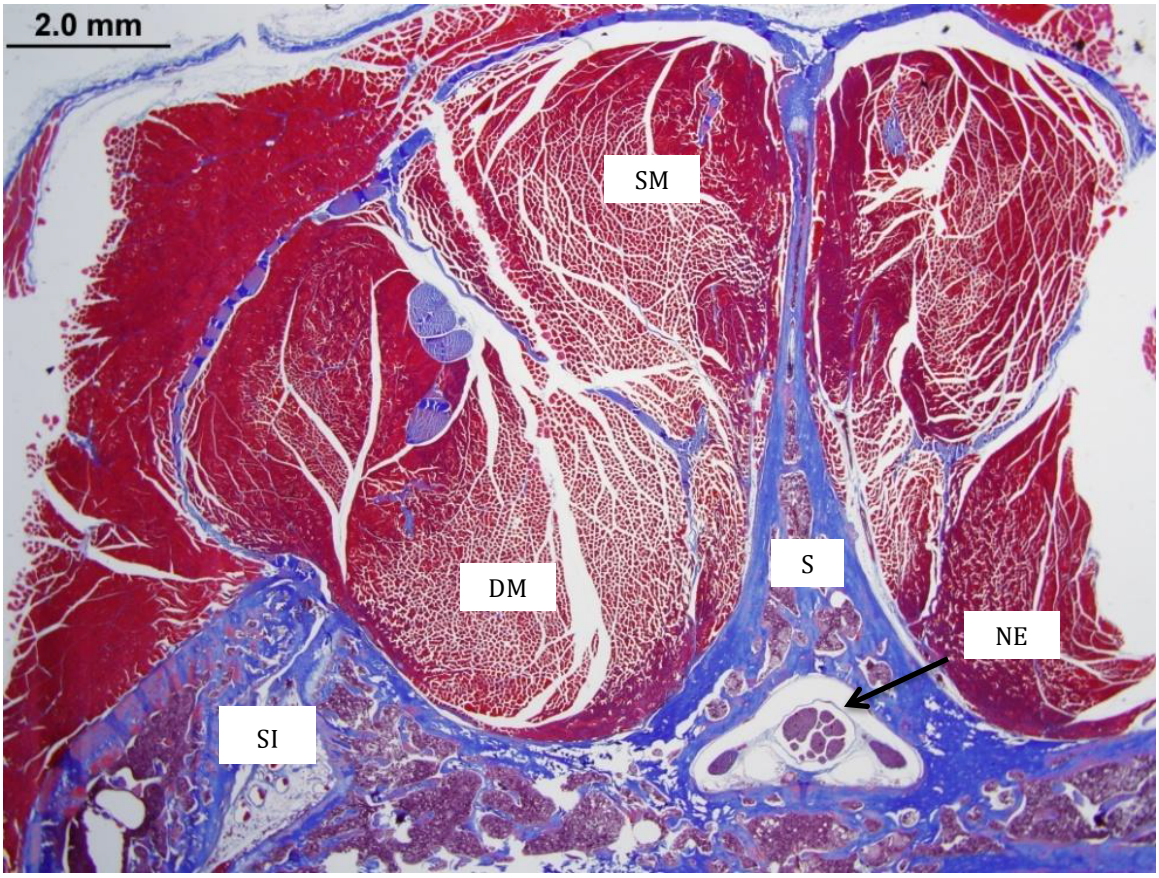

Figure 4.5: Histology of nonsurgical control rat.
Superficial epaxial muscles (SM), deep epaxial muscles (DM), neural elements (NE), first sacral vertebra posterior elements (S), ileosacral articulation (SI). Masson trichrome stain technique, 20X magnification.

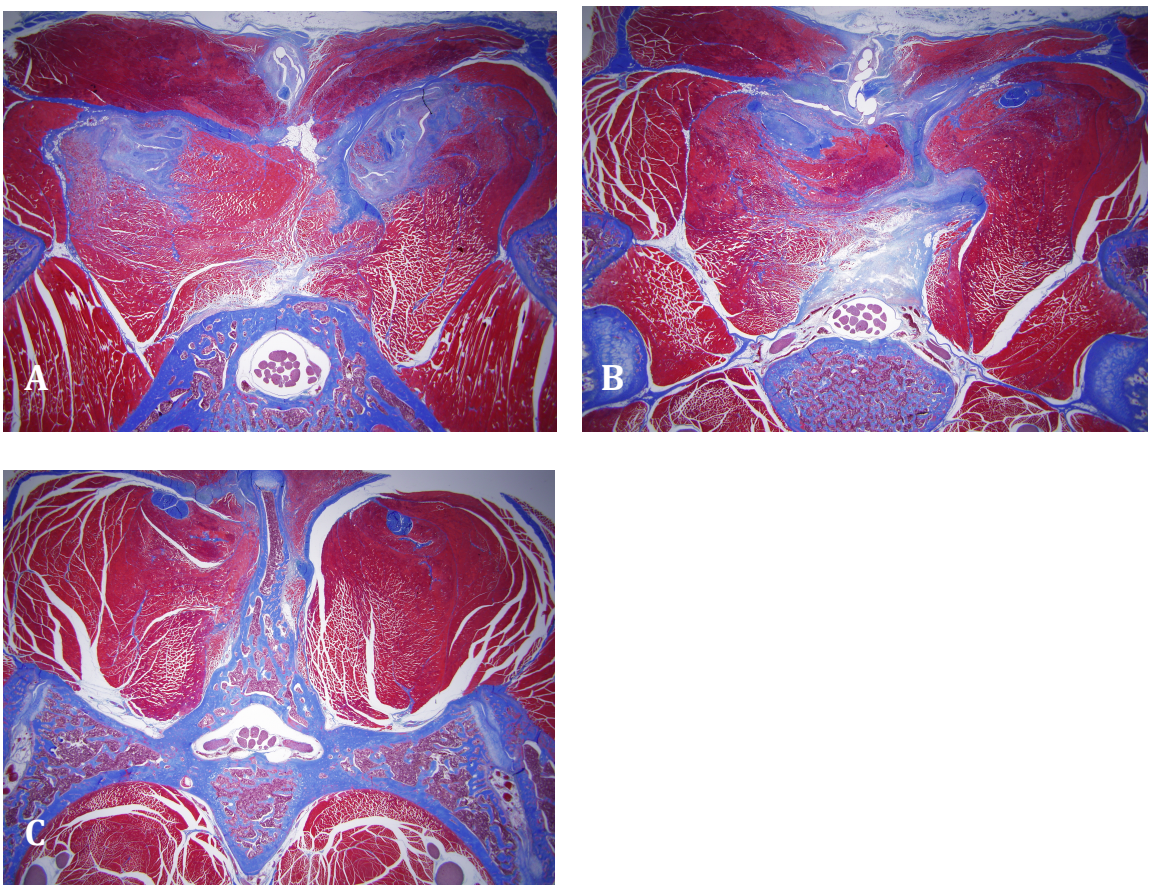

Figure 4.6: Histology of normal saline treated rat following laminectomy.
High grade (grade 3) epidural fibrosis at laminectomy site. **(A)** Cephalad to the laminectomy; **(B)** At the laminectomy site; **(C)** Caudal to the laminectomy site. Masson trichrome stain technique, 20X magnification.

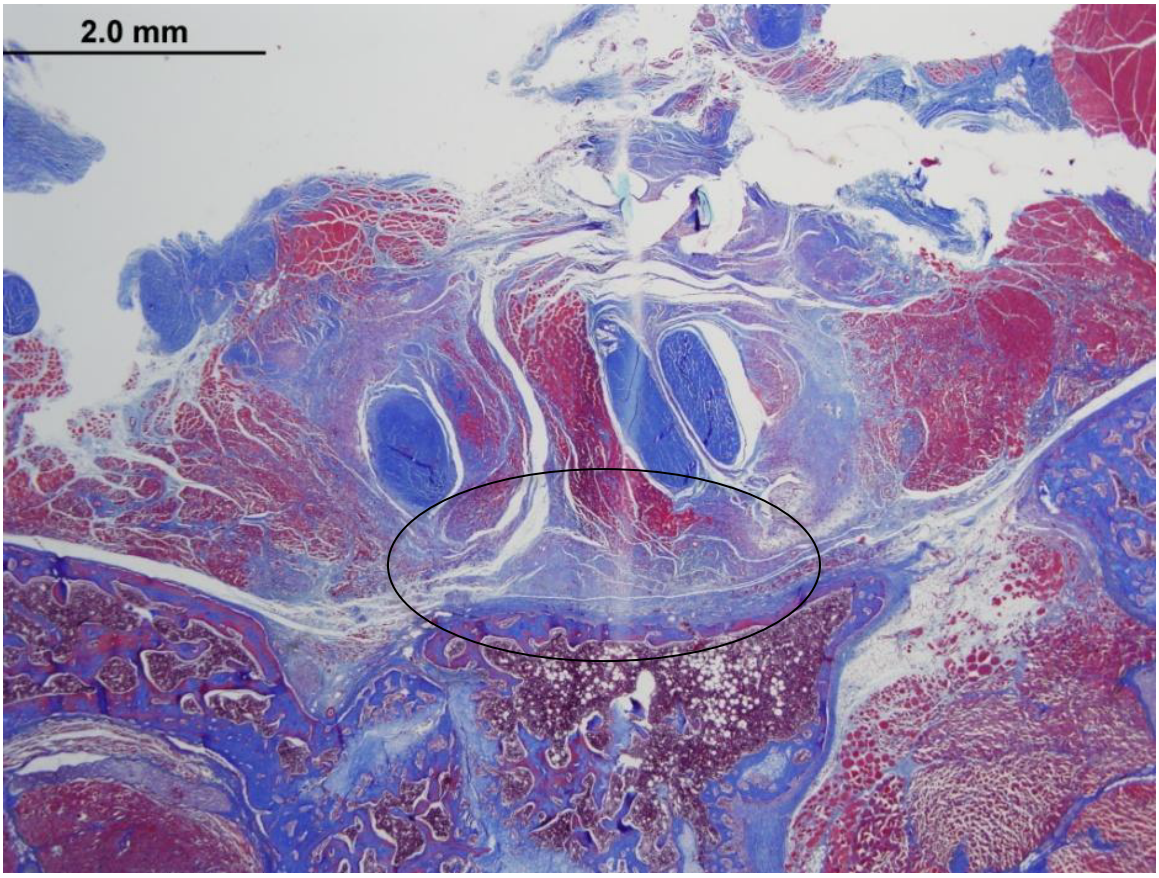

Figure 4.7: Histology of rat having undergone laminectomy, severe fibrosis at laminectomy site.
At laminectomy site (circled), neural elements are indistinguishable from fibrotic material. Masson trichrome technique, 20X magnification.

4.4.4 Observations in alanyl-glutamine treated animals

Similar to normal saline treated rats, alanyl-glutamine treated rats undergoing laminectomy demonstrated exuberant fibrosis in the epidural, deep and superficial epaxial layers (Figure 4.8).

Of the 26 rats in the alanyl-glutamine treatment group, 20 rats had grade 4 and six had grade 3 epidural fibrosis. The quality or maturity of the fibrotic material did not appear qualitatively different from that observed in the surgical control (normal saline) group. When comparing rats treated with alanyl-glutamine (n=26) and normal saline control rats (n=25), there was no statistically significant difference in the grade of epidural fibrosis (p=0.83, Figure 4.9).

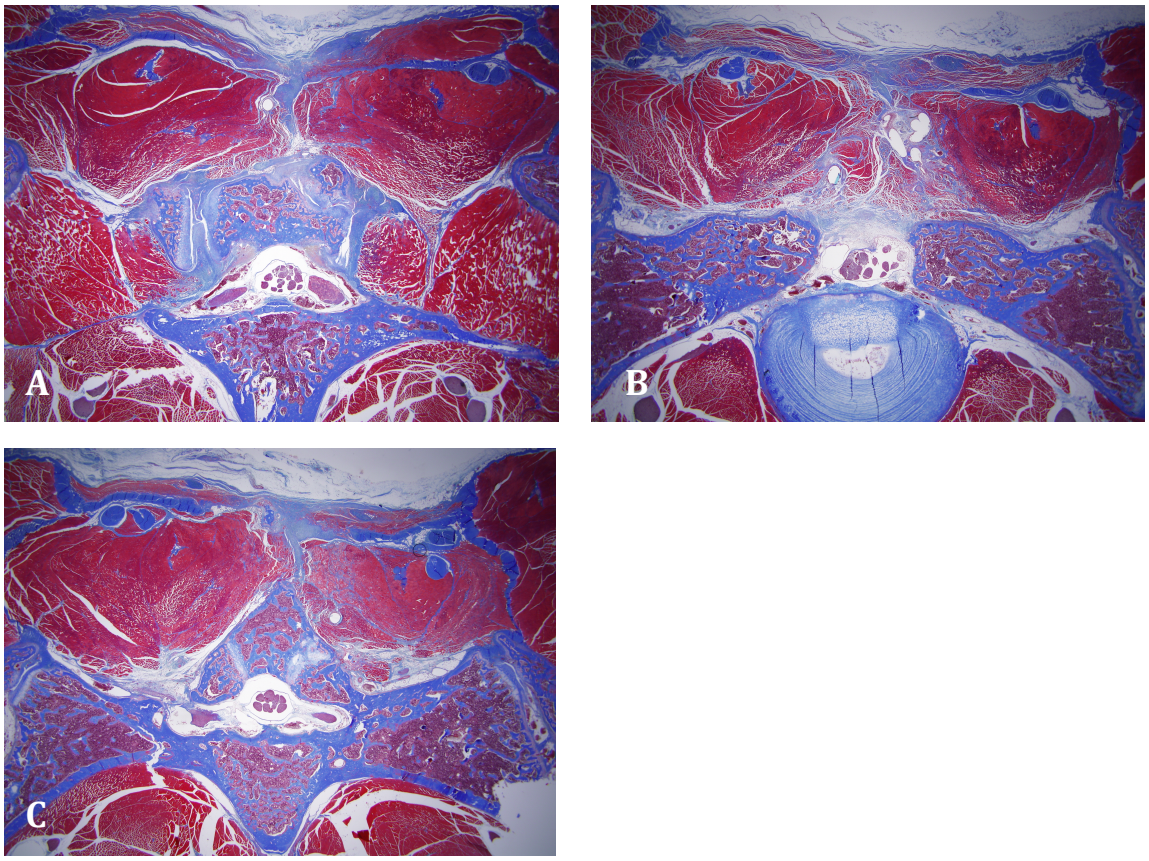

Figure 4.8: Histology of alanyl-glutamine treated rat following laminectomy. High grade (grade 3) epidural fibrosis at laminectomy site. **(A)** Cephalad to the laminectomy; **(B)** At the laminectomy site; **(C)** Caudal to the laminectomy site. Masson trichrome technique, 20X magnification.

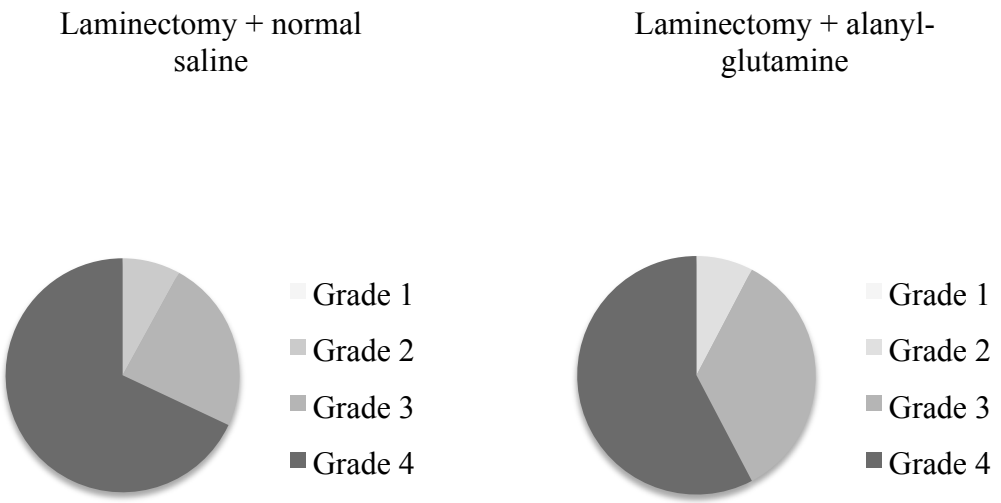

Figure 4.9: Histological grade of epidural fibrosis in surgical control and alanyl-glutamine treatment groups.
When comparing rats treated with alanyl-glutamine (n=26) and normal saline control rats (n=25), there was no statistically significant difference in the grade of epidural fibrosis (p=0.83).

Groups were also combined as subsets of 'low grade' (grade 1 and 2) and 'high grade' (grade 3 and 4) and compared. When comparing rats treated with normal saline, there was no statistically significant difference between the low and high-grade subsets (p=0.89, Figure 4.10).

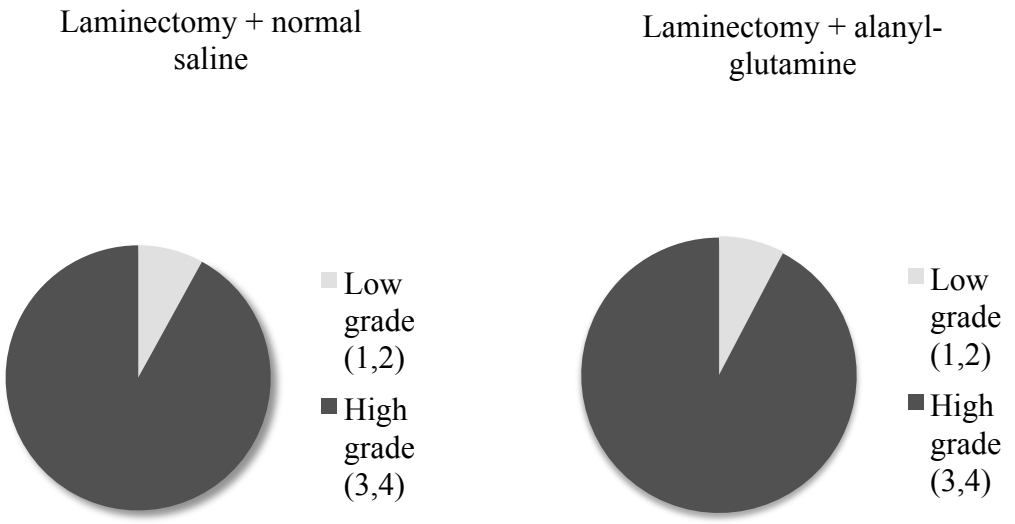

Figure 4.10: Histological grade of epidural fibrosis in "low grade" and "high grade" subsets.
When comparing rats treated with normal saline, there was no statistically significant difference between the low and high-grade subsets (p=0.89).

4.5 Intra- and inter-observer reliability of the grading scheme

Based on 104 pairs of independent observations performed by two raters, the intra-rater reliability kappa value was 0.71 ($p<0.001$), 95% CI (0.60, 0.80). The inter-rater reliability kappa value was 0.62 ($p<0.001$), 95% CI (0.47, 0.77).

5. DISCUSSION

5.1 Effectiveness of the rat laminectomy model for studying epidural fibrosis

Due to availability, low cost and ease of care, the rat laminectomy model has been popular in studies investigating therapeutic interventions for epidural fibrosis. All models have caveats, and for the rat laminectomy model, these are primarily anatomical concerns. The small size of the animal, thin lamina, small epidural space and low set conus medullaris (the terminal end of the spinal cord) increase the technical challenge of surgical interventions on the spine[116]. Nevertheless, the model continues to be successfully used in studies that employ histological techniques to evaluate potential therapeutic interventions[240-243].

The results of this study confirm the suitability of the rat laminectomy model for studying epidural fibrosis. There was a learning curve associated with the operative procedure, with the first fifty percent of procedures taking significantly longer than the latter half. However, the surgical procedure was performed with minimal postoperative complications, and without devastating neurologic sequelae. When compared to rats that did not undergo surgery, rats that underwent surgical laminectomy demonstrated exuberant fibrotic proliferation in the epidural space. The significant response seen would be suitable for testing potent antifibrotic agents. It would be less suitable for testing less potent agents for which a small effect may be masked by the overall exuberant fibrotic response.

5.2 Lack of efficacy of alanyl-glutamine

Based on presumed efficacy of alanyl-glutamine in reducing peritoneal adhesions in a post-surgical rat model[232], I had hypothesized a similar effect on reduction of epidural fibrosis following laminectomy. Although the topical application of alanyl-glutamine appeared safe with no observed systemic complications, there was no statistically significant difference in grade of epidural fibrosis in alanyl-glutamine treated rats compared to those treated with normal saline. The results of this study suggest that

direct application of an alanyl-glutamine solution to the epidural space does not have any effect on reducing epidural fibrosis at a histological level.

5.3 Possible explanations for lack of efficacy

Lack of effect may be related to the environment, the model, potency of the agent, treatment dose and duration of exposure. Each of these factors may be wholly responsible for or contribute to the lack of observed effect.

5.3.1 Environmental differences

The initial research that suggested a positive effect of alanyl-glutamine on reducing fibrosis utilized a rat peritoneal sepsis model[232]. In this model, the inflammatory cascade was initiated as a result of a cecal ligation puncture, which resulted in both surgical trauma and bowel necrosis with fecal contamination and subsequent infection. This is vastly different environment than that of the post-operative peridural region. Additionally, the peritoneal model involves relatively little dissection and has negligible hematoma formation. Given these differences, there may be alterations in the mechanisms of tissue repair and remodeling.

In addition to the major differences in the models employed, there are dissimilarities in the macro- and microenvironments of the peritoneal cavity and the epidural space that may account for differences in observed effect of alanyl-glutamine. In contrast to the closed space of the peritoneal cavity, the treatment substance may leak from the surgical field into dissection planes and from the wound itself. The peritoneum also has a greater absorptive surface, and the type and number of glutamine responsive cells may affect the efficiency of glutamine uptake and utilization.

5.3.2 Sensitivity of the model

It is possible that alanyl-glutamine may have an effect that is not detectable using this model. The rats in this study demonstrated exuberant epidural fibrosis to the extent that it would be difficult to precisely quantify. Models employing more sensitive techniques such as quantification of absolute numbers of fibroblasts or inflammatory cells though microscopy[111 113 117 233] or immunohistochemistry[38 113 124] may be more successful. Based on discussions with a pathologist, we do not feel that either technique would be beneficial in our specimens due to the extent of fibrosis observed.

5.3.3 Delivery, dose and potency of the treatment substance

Other factors to consider include the method of delivery, treatment dose and potency of the antifibrotic agent being studied. While the extent of uptake and utilization of intravenously supplied alanyl-glutamine in the rat has been studied[237 244], the extent of absorption via topical administration is unknown. It is also unknown whether topical administration of alanyl-glutamine to the wound is the optimal delivery method for prevention of epidural fibrosis, and further study of intravenous administration may be warranted.

The concentration of alanyl-glutamine used in this study approaches the maximum soluble concentration[237], and the treatment dose was selected based on previous research[232 235 236]. The minimum effective tissue concentration of alanyl-glutamine required to exert an effect on fibrosis formation is unknown. In order to further define this and optimal dose, *in vitro* studies utilizing inflammatory cells may be designed. If there is no observed effect of alanyl-glutamine at maximal soluble concentration, multiple treatments or prolonged exposure may be considered.

5.3.4 Duration of exposure

The critical period of inflammatory cell trafficking into the healing wound is the early hours post-injury. If alanyl-glutamine were to be effective, it would have to be present at minimally effective concentrations throughout this period. While the half-life of alanyl-glutamine applied to the tissues is unknown, the half-life of intravenously supplied alanyl-glutamine is relatively short (range 5-16 minutes)[237 245]. Even with the prolonged retention in the closed space of the peritoneal cavity, it is difficult to imagine that such a short exposure to alanyl-glutamine is able to exert any prolonged effect. Therefore, it would be desirable to increase duration of exposure with strategies to retain the solution in the surgical field and to control its release throughout the critical inflammatory period.

5.4 Concern for reliability of the grading scheme

The rating scale utilized in this study relies on subjective interpretation of histopathology. It is therefore important to consider the validity of the scoring system by

assessing agreement of observers on subsequent re-observation (intra-rater reliability) and between different observers on the same observation (inter-rater reliability).

In this study, the intra-rater reliability kappa value was 0.71 ($p<0.001$), 95% CI (0.60, 0.80). The inter-rater reliability kappa value was 0.62 ($p<0.001$), 95% CI (0.47-0.77).

There are arbitrary guidelines in the literature classifying the degree of reliability. Landis and Koch suggested that kappa may be interpreted as: values <0.0 indicating no agreement, 0.0-0.20 as slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial and 0.81-1.00 as almost perfect agreement[246]. Applying these guidelines, the calculated kappa values suggest a "substantial" level of intra- and inter-rater agreement.

However, concerns arise when considering the lower endpoint of the calculated confidence interval for kappa. While still falling into "moderate" strength of agreement according to Landis and Koch, the values suggest a significant amount of inconsistency in individual interpretation, as well as across multiple data collectors. Minimum acceptable levels of agreement in health research have not been established, but many authors suggest a minimum kappa value of 0.80[247]. Improved definitions for each grade of epidural fibrosis may improve the intra-rater reliability. The low inter-rater reliability may reflect the observations of a relatively inexperienced observer, and could be improved with observer training.

5.5 Future directions

Epidural fibrosis after spinal surgery is inevitable. The formation of post-surgical fibrosis is linked to the inflammatory cascade that occurs after injury, and the key to its prevention lies in successful modulation of this pathway.

An understanding of the basic mechanisms by which glutamine acts to reduce peritoneal adhesions is lacking. Resources may be better utilized to define this mechanism, and further studies can be designed to exploit those specific mechanisms. Data outlining the topical absorption and *in vivo* tissue half-life of alanyl-glutamine would also be useful to appropriately target delivery of maximal concentrations of alanyl-glutamine. Future research may also explore controlled elution of the treatment substance in an animal model. Only after repeated positive results in controlled animal studies can any potential treatment be considered for human trials.

Currently, there is no treatment which has been consistently proven effective in human trials and which is in routine clinical use to prevent epidural fibrosis following spine surgery. With no effective therapy available, prevention remains the goal, and surgeons should aim for minimal tissue disruption with meticulous hemostasis.

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